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(54) Title: HLA BINDING PEPTIDES AND THEIR USES			
(57) Abstract The present invention provides peptide compositions capable of binding glycoproteins encoded by HLA, HLA-B, and HLA-C alleles and inducing T cell activation in T cells restricted by the HLA allele. The peptides are useful to elicit an immune response against a desired antigen.			

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HLA Binding Peptides and Their Uses

CROSS REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of application U.S. Serial No. 08/344,824, filed November 23, 1994, which is a continuation-in-part of application U.S. Serial No. 08/278,634 filed July 21, 1994, both of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

The present invention relates to compositions and methods for preventing, treating or diagnosing a number of pathological states such as viral diseases and cancers. In particular, it provides novel peptides capable of binding selected major histocompatibility complex (MHC) molecules and inducing an immune response.

MHC molecules are classified as either Class I or Class II molecules. Class II MHC molecules are expressed primarily on cells involved in initiating and sustaining immune responses, such as T lymphocytes, B lymphocytes, macrophages, etc. Class II MHC molecules are recognized by helper T lymphocytes and induce proliferation of helper T lymphocytes and amplification of the immune response to the particular immunogenic peptide that is displayed. Class I MHC molecules are expressed on almost all nucleated cells and are recognized by cytotoxic T lymphocytes (CTLs), which then destroy the antigen-bearing cells. CTLs are particularly important in tumor rejection and in fighting viral infections. The CTL recognizes the antigen in the form of a peptide fragment bound to the MHC class I molecules rather than the intact foreign antigen itself. The antigen must normally be endogenously synthesized by the cell, and a portion of the protein antigen is degraded into small peptide fragments in the cytoplasm. Some of these small peptides translocate into a pre-Golgi compartment and interact with class I heavy chains to facilitate proper folding and association with the subunit $\beta 2$ microglobulin. The peptide-MHC class I complex is then routed to the cell surface for expression and potential recognition by specific CTLs.

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The MHC class I antigens are encoded by the HLA-A, B, and C loci. HLA-A and HLA-B antigens are expressed at the cell surface at approximately equal densities, whereas the expression of HLA-C is significantly lower (perhaps as much as 10-fold lower). Each of these loci have a number of alleles.

Specific motifs for several of the major HLA-A alleles (copending U.S. Patent Applications 08/159,339 and 08/205,713, referred to here as the copending applications) and HLA-B alleles have been described. Several authors (Melief, *Eur. J. Immunol.*, 21:2963-2970 (1991); Bevan, et al., *Nature* 353:852-955 (1991)) have provided preliminary evidence that class I binding motifs can be applied to the identification of potential immunogenic peptides in animal models. Strategies for identification of peptides or peptide regions capable of interacting with multiple MHC alleles has been described in the literature.

Because human population groups, including racial and ethnic groups, have distinct patterns of distribution of HLA alleles it will be of value to identify motifs that describe peptides capable of binding more than one HLA allele, so as to achieve sufficient coverage of all population groups. The present invention addresses these and other needs.

SUMMARY OF THE INVENTION

The present invention provides compositions comprising immunogenic peptides having binding motifs for HLA alleles. The immunogenic peptides are about 9 to 10 residues in length and comprise conserved residues at certain positions such as a proline at position 2 and an aromatic residue (e.g., Y, W, F) or hydrophobic residue (e.g., L, I, V, M, or A) at the carboxy terminus. In particular, an advantage of the peptides of the invention is their ability to bind to two or more different HLA alleles.

The present invention defines positions within a motif enabling the selection of peptides that will bind efficiently to more than one HLA-A, HLA-B or HLA-C alleles. Epitopes possessing the motif of the immunogenic peptides have been identified on potential target antigens including hepatitis B core and surface antigens (HBVc, HBVs), hepatitis C antigens, Epstein-Barr virus antigens, and human immunodeficiency type-1 virus (HIV1). Thus, the invention further provides immunogenic peptides comprising sequences of target antigens.

The peptides of the invention are useful in pharmaceutical compositions for both *in vivo* and *ex vivo* therapeutic and diagnostic applications.

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Definitions

The term "peptide" is used interchangeably with "oligopeptide" in the present specification to designate a series of residues, typically L-amino acids, connected one to the other typically by peptide bonds between the alpha-amino and carbonyl groups of adjacent amino acids. The oligopeptides of the invention are less than about 15 residues in length and usually consist of between about 8 and about 11 residues, preferably 9 or 10 residues.

An "immunogenic peptide" is a peptide which comprises an allele-specific motif such that the peptide will bind an MHC molecule and induce a CTL response. Immunogenic peptides of the invention are capable of binding to an appropriate HLA molecule and inducing a cytotoxic T cell response against the antigen from which the immunogenic peptide is derived.

A "conserved residue" is a conserved amino acid occupying a particular position in a peptide motif typically one where the MHC structure may provide a contact point with the immunogenic peptide. One to three, typically two, conserved residues within a peptide of defined length defines a motif for an immunogenic peptide. These residues are typically in close contact with the peptide binding groove, with their side chains buried in specific pockets of the groove itself.

The term "motif" refers to the pattern of residues in a peptide of defined length, usually about 8 to about 11 amino acids, which is recognized by a particular MHC allele. The peptide motifs are typically different for each human MHC allele.

The term "supermotif" refers to motifs that, when present in an immunogenic peptide, allow the peptide to bind more than one HLA antigen. The supermotif preferably is recognized by at least one HLA allele having a wide distribution in the human population, preferably recognized by at least two alleles, more preferably recognized by at least three alleles, and most preferably recognized by more than three alleles.

The phrases "isolated" or "biologically pure" refer to material which is substantially or essentially free from components which normally accompany it as found in its native state. Thus, the peptides of this invention do not contain materials normally associated with their *in situ* environment, e.g., MHC I molecules on antigen presenting cells. Even where a protein has been isolated to a homogenous or dominant band, there are trace contaminants in the range of 5-10% of native protein which co-purify with the

desired protein. Isolated peptides of this invention do not contain such endogenous co-purified protein.

The term "residue" refers to an amino acid or amino acid mimetic incorporated in an oligopeptide by an amide bond or amide bond mimetic.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows binding motifs for peptides capable of binding HLA alleles sharing the B7-like specificity.

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Figure 2 shows the B7-like cross-reactive motif.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to the determination of allele-specific peptide motifs for human Class I MHC (sometimes referred to as HLA) allele subtypes. In particular, the invention provides motifs that are common to peptides bound by more than one HLA allele. By a combination of motif identification and MHC-peptide interaction studies, peptides useful for peptide vaccines have been identified.

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Following the methods described in the copending applications noted above, certain peptides capable of binding at multiple HLA alleles which possess a common motif have been identified. The motifs of those peptides can be characterized as follows: N-XPXXXXXX(AVILM)-C; N-XPXXXXXX(AVILM)-C; N-XPXXXXXX(FWY)-C; and N-XPXXXXXX(FWY)-C. Motifs that are capable of binding at multiple alleles are referred to here as "supermotifs." The particular supermotifs above are specifically called "B7-like-supermotifs."

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Immunogenic peptides of the invention are typically identified using a computer to scan the amino acid sequence of a desired antigen for the presence of the supermotifs. Examples of antigens include viral antigens and antigens associated with cancer. An antigen associated with cancer is an antigen, such as a melanoma antigen, that is characteristic of (i.e., expressed by) cells in a malignant tumor but not normally expressed by healthy cells. Examples of suitable antigens particularly include hepatitis B core and surface antigens (HBVc, HBVs) hepatitis C antigens, Epstein-Barr virus antigens, and human immunodeficiency virus (HIV) antigens, and also include prostate

specific antigen (PSA), melanoma antigens (e.g., MAGE-1), and human papilloma virus (HPV) antigens; this list is not intended to exclude other sources of antigens.

Peptides comprising the supermotif sequences, including those found in proteins from potential antigenic sources are synthesized and then tested for their ability to bind to the appropriate MHC molecules in a variety of assays. The assays may use, for example, purified class I molecules and radioiodinated peptides. Alternatively, binding to cells expressing empty class I molecules can be detected by, for instance, immunofluorescent staining and flow microfluorimetry. Those peptides that bind to the class I molecule may be further evaluated for their ability to serve as targets for CTLs derived from infected or immunized individuals, as well as for their capacity to induce primary *in vitro* or *in vivo* CTL responses that can give rise to CTL populations capable of reacting with virally infected target cells or tumor cells as therapeutic agents.

Recent evidence suggests however, that high affinity MHC binders might be, in most instances, immunogenic, suggesting that peptide epitopes might be selected on the basis of MHC binding alone.

Peptides comprising the supermotif sequences can be identified, as noted above, by screening potential antigenic sources. Useful peptides can also be identified by synthesizing peptides with systematic or random substitution of the variable residues in the supermotif, and testing them according to the assays provided. As demonstrated below, it is useful to refer to the sequences of the target HLA molecule, as well.

The nomenclature used to describe peptide compounds follows the conventional practice wherein the amino group is presented to the left (the N-terminus) and the carboxyl group to the right (the C-terminus) of each amino acid residue. In the formulae representing selected specific embodiments of the present invention, the amino- and carboxyl-terminal groups, although not specifically shown, are in the form they would assume at physiologic Ph values, unless otherwise specified. In the amino acid structure formulae, each residue is generally represented by standard three letter or single letter designations. The L-form of an amino acid residue is represented by a capital single letter or a capital first letter of a three-letter symbol, and the D-form for those amino acids having D-forms is represented by a lower case single letter or a lower case three letter symbol. Glycine has no asymmetric carbon atom and is simply referred to as "Gly" or G. The letter X in a motif represents any of the 20 amino acids found in Table 1, as well non-naturally occurring amino acids or amino acid mimetics. Brackets

surrounding more than one amino acid indicates that the motif includes any one of the amino acids. For example, the supermotif "N-XPXXXXXX(AVILM)-C" includes each of the following peptides: N-XPXXXXXXA-C, N-XPXXXXXXV-C, N-XPXXXXXXI-C, N-XPXXXXXXL-C, and N-XPXXXXXXM-C.

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For peptide-based vaccines, the peptides of the present invention preferably comprise a motif (Table 2) shows the distribution of certain HLA alleles in human populations..

TABLE 1

	<u>Original Residue</u>	<u>Exemplary Substitution</u>
5	Ala	ser
	Arg	lys
	Asn	gln
10	Asp	glu
	Cys	ser
15	Gln	asn
	Glu	asp
	Gly	pro
20	His	arg; lys
	Ile	leu; val; met
25	Leu	ile; val; met
	Lys	arg
	Met	leu; ile; val
30	Phe	tyr; trp
	Ser	thr
35	Thr	ser
	Trp	tyr; phe
	Tyr	trp; phe
40	Val	ile; leu; met

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TABLE 2
Summary of Population Coverage by Currently Available Assays

Phenotypic (Allelic) Frequency							
Antigen	HLA Allele	Cell Line(s)	Caucasian	Negro	Japanese	Chinese	Hispanic
A1	A*0101	Steinlin	28.6	10.1	1.4	9.2	10.1
A2.1	A*0201	JY	45.8	30.3	42.4	54.0	43.0
A3.2	A*0301	GM3107	20.6	16.3	1.2	7.1	14.8
A11	A*1101	BVR	9.9	3.8	19.7	33.1	7.3
A24	A*2401	KT3	16.8	8.8	58.1	32.9	26.7
All A			88.9	59.8	91.6	94.6	80.2
B7	B*0701	GM3107	17.7	15.5	9.6	6.9	11.8
B8	B*0801	Steinlin	18.1	6.3	0.0	3.6	9.0
B27	B*2705	LG2	7.5	2.6	0.8	3.4	4.9
B35	B*3503	BHM	15.4	14.8	15.4	9.8	28.1
B54	B*5401	KT3	0.0	0.0	12.4	8.6	0.0
All B			51.9	36.5	35.6	30.2	48.7
Cw6	Cw0601	C1R	17.6	13.7	2.2	19.0	12.2
TOTAL			95.7	76.5	94.7	96.6	91.0

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For assays of peptide-HLA interactions (e.g., quantitative binding assays) cells with defined MHC molecules are useful. A large number of cells with defined MHC molecules, particularly MHC Class I molecules, are known and readily available.

For example, human EBV-transformed B cell lines have been shown to be excellent sources for the preparative isolation of class I and class II MHC molecules.

Well-characterized cell lines are available from private and commercial sources, such as American Type Culture Collection ("Catalogue of Cell Lines and Hybridomas," 6th edition (1988) Rockville, Maryland, U.S.A.); National Institute of General Medical Sciences 1990/1991 Catalog of Cell Lines (NIGMS) Human Genetic Mutant Cell

Repository, Camden, NJ; and ASHI Repository, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115. Cell lines suitable as sources for various HLA-A alleles are described in the copending applications. Table 3 lists some B cell lines suitable for use as sources for HLA-B and HLA-C alleles, which are particularly useful

in the present invention. All of these cell lines can be grown in large batches and are therefore useful for large scale production of MHC molecules. One of skill will recognize that these are merely exemplary cell lines and that many other cell sources can be employed.

TABLE 3
HUMAN CELL LINES (HLA-B and HLA-C SOURCES)

5		
	HLA-B allele	B cell line
	B1801	DVCAF
	B3503	EHM
10	B0701	GM3107
	B1401	LWAGS
	B5101	KAS116
	B5301	AMAI
	B0801	MAT
15	B2705	LG2
	B5401	KT3
	B1302	CBUF
	B4403	PITOUT
	B3502	TISI
20	B3501	BUR
	B4001	LB
	HLA-C allele	B cell line
25	Cw0601	C1R

30 In the typical case, immunoprecipitation is used to isolate the desired allele. A number of protocols can be used, depending upon the specificity of the antibodies used. For example, allele-specific mAb reagents can be used for the affinity purification of the HLA-A, HLA-B, and HLA-C molecules. Monoclonal antibodies available for isolating various HLA molecules include those listed in Table 4. Affinity columns prepared with these mAbs using standard techniques are used to purify the
35 respective HLA allele products.

TABLE 4

ANTIBODY REAGENTS

5	anti-HLA	Name
	HLA-A2	BB7.2
10	HLA-A1	12/18
	HLA-A3	GAPA3 (ATCC, HB122)
15	HLA-11,24.1	A11.1M (ATCC, HB164)
	HLA-A,B,C	W6/32 (ATCC, HB95)
	monomorphic	B9.12.1
20	HLA-B,C	B.1.23.2
	monomorphic	

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The capacity to bind MHC Class I molecules is measured in a variety of different ways. One means is a Class I molecular binding assay as described in Example 2, below. Other alternatives described in the literature include inhibition of antigen presentation (Sette, et al., *J. Immunol.* 141:3893 (1991)), *in vitro* assembly assays (Townsend, et al., *Cell* 62:285 (1990)), and FACS based assays using mutated cells, such as RMA.S (Melief, et al., *Eur. J. Immunol.* 21:2963 (1991)).

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Next, peptides that test positive in the MHC class I binding assay are assayed for the ability of the peptides to induce specific CTL responses *in vitro*. For instance, antigen-presenting cells that have been incubated with a peptide can be assayed for the ability to induce CTL responses in responder cell populations. Antigen-presenting cells can be normal cells such as peripheral blood mononuclear cells or dendritic cells (Inaba, et al., *J. Exp. Med.* 166:182 (1987); Boog, *Eur. J. Immunol.* 18:219 (1988)). Alternatively, transgenic mice comprising an appropriate HLA transgene can be used to assay the ability of a peptide to induce a response in cytotoxic T lymphocytes essentially as described in copending U.S. Patent Application No.

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08/205,713.

Alternatively, mutant mammalian cell lines that are deficient in their ability to load class I molecules with internally processed peptides, such as the mouse cell lines RMA-S (Kärre, et al., *Nature*, 319:675 (1986); Ljunggren, et al., *Eur. J. Immunol.* 21:2963-2970 (1991)), and the human T cell hybridoma, T-2 (Cerundolo, et al., *Nature* 345:449-452 (1990)) and which have been transfected with the appropriate human class I genes are conveniently used, when peptide is added to them, to test for the capacity of the peptide to induce *in vitro* primary CTL responses. Other eukaryotic cell lines which could be used include various insect cell lines such as mosquito larvae (ATCC cell lines CCL 125, 126, 1660, 1591, 6585, 6586), silkworm (ATCC CRL 8851), armyworm (ATCC CRL 1711), moth (ATCC CCL 80) and *Drosophila* cell lines such as a Schneider cell line (see Schneider *J. Embryol. Exp. Morphol.* 27:353-365 (1927)).

Peripheral blood lymphocytes are conveniently isolated following simple venipuncture or leukapheresis of normal donors or patients and used as the responder cell sources of CTL precursors. In one embodiment, the appropriate antigen-presenting cells are incubated with 10-100 μ M of peptide in serum-free media for 4 hours under appropriate culture conditions. The peptide-loaded antigen-presenting cells are then incubated with the responder cell populations *in vitro* for 7 to 10 days under optimized culture conditions. Positive CTL activation can be determined by assaying the cultures for the presence of CTLs that kill radiolabeled target cells, both specific peptide-pulsed targets as well as target cells expressing endogenously processed form of the relevant virus or tumor antigen from which the peptide sequence was derived.

Specificity and MHC restriction of the CTL is determined by testing against different peptide target cells expressing appropriate or inappropriate human MHC class I. The peptides that test positive in the MHC binding assays and give rise to specific CTL responses are referred to herein as immunogenic peptides.

The immunogenic peptides can be prepared synthetically, or by recombinant DNA technology. Although the peptide will preferably be substantially free of other naturally occurring host cell proteins and fragments thereof, in some embodiments the peptides can be synthetically conjugated to native fragments or particles.

The polypeptides or peptides can be a variety of lengths, either in their

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neutral (uncharged) forms or in forms which are salts, and either free of modifications such as glycosylation, side chain oxidation, or phosphorylation or containing these modifications, subject to the condition that the modification not destroy the biological activity of the polypeptides as herein described.

Desirably, the peptide will be as small as possible while still maintaining substantially all of the biological activity of the large peptide. When possible, it may be desirable to optimize peptides of the invention to a length of 9 or 10 amino acid residues, commensurate in size with endogenously processed viral peptides or tumor cell peptides that are bound to MHC class I molecules on the cell surface.

Peptides having the desired activity may be modified as necessary to provide certain desired attributes, e.g., improved pharmacological characteristics, while increasing or at least retaining substantially all of the biological activity of the unmodified peptide to bind the desired MHC molecule and activate the appropriate T cell. For instance, the peptides may be subject to various changes, such as substitutions, either conservative or non-conservative, where such changes might provide for certain advantages in their use, such as improved MHC binding. By conservative substitutions is meant replacing an amino acid residue with another which is biologically and/or chemically similar, e.g., one hydrophobic residue for another, or one polar residue for another. The substitutions include combinations such as Gly, Ala; Val, Ile, Leu, Met; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. The effect of single amino acid substitutions may also be probed using D-amino acids. Such modifications may be made using well known peptide synthesis procedures, as described in e.g., Merrifield, *Science* 232:341-347 (1986), Barany and Merrifield, *The Peptides*, Gross and Meienhofer, eds. (N.Y., Academic Press), pp. 1-284 (1979); and Stewart and Young, *Solid Phase Peptide Synthesis*, (Rockford, Ill., Pierce), 2d Ed. (1984), incorporated by reference herein.

The peptides can also be modified by extending or decreasing the compound's amino acid sequence, e.g., by the addition or deletion of amino acids. The peptides or analogs of the invention can also be modified by altering the order or composition of certain residues, it being readily appreciated that certain amino acid residues essential for biological activity, e.g., those at critical contact sites or conserved residues, may generally not be altered without an adverse effect on biological activity. The non-critical amino acids need not be limited to those naturally occurring in proteins, such as L- α -amino acids, or their D-isomers, but may include non-protein amino acids as

well, such as β - γ - δ -amino acids, as well as many derivatives of L- α -amino acids.

Typically, a series of peptides with single amino acid substitutions are employed to determine the effect of electrostatic charge, hydrophobicity, etc. on binding. For instance, a series of positively charged (e.g., Lys or Arg) or negatively charged (e.g., Glu) amino acid substitutions are made along the length of the peptide revealing different patterns of sensitivity towards various MHC molecules and T cell receptors. In addition, multiple substitutions using small, relatively neutral moieties such as Ala, Gly, Pro, or similar residues may be employed. The substitutions may be homo-oligomers or hetero-oligomers. The number and types of residues which are substituted or added depend on the spacing necessary between essential contact points and certain functional attributes which are sought (e.g., hydrophobicity versus hydrophilicity). Increased binding affinity for an MHC molecule or T cell receptor may also be achieved by such substitutions, compared to the affinity of the parent peptide. In any event, such substitutions should employ amino acid residues or other molecular fragments chosen to avoid, for example, steric and charge interference which might disrupt binding.

Amino acid substitutions are typically of single residues. Substitutions, deletions, insertions or any combination thereof may be combined to arrive at a final peptide. Substitutional variants are those in which at least one residue of a peptide has been removed and a different residue inserted in its place. Such substitutions generally are made in accordance with Table 1 when it is desired to finely modulate the characteristics of the peptide.

Substantial changes in function (e.g., affinity for MHC molecules or T cell receptors) are made by selecting substitutions that are less conservative than those in Table 1, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution, for example as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in peptide properties will be those in which (a) hydrophilic residue, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g. glutamyl or aspartyl; or (d) a residue having a bulky side

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chain, e.g. phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine.

The peptides may also comprise isosteres of two or more residues in the immunogenic peptide. An isostere as defined here is a sequence of two or more residues that can be substituted for a second sequence because the steric conformation of the first sequence fits a binding site specific for the second sequence. The term specifically includes peptide backbone modifications well known to those skilled in the art. Such modifications include modifications of the amide nitrogen, the α -carbon, amide carbonyl, complete replacement of the amide bond, extensions, deletions or backbone crosslinks. See, generally, Spatola, *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins*, Vol. VII (Weinstein ed., 1983).

Modifications of peptides with various amino acid mimetics or D-amino acids, for instance at the N- or C- termini, are particularly useful in increasing the stability of the peptide *in vivo*. Stability can be assayed in a number of ways. For instance, peptidases and various biological media, such as human plasma and serum, have been used to test stability. See, e.g., Verhoef et al., *Eur. J. Drug Metab. Pharmacokin.* 11:291-302 (1986). Half life of the peptides of the present invention is conveniently determined using a 25% human serum (v/v) assay. The protocol is generally as follows. Pooled human serum (Type AB, non-heat inactivated) is delipidated by centrifugation before use. The serum is then diluted to 25% with RPMI tissue culture media and used to test peptide stability. At predetermined time intervals a small amount of reaction solution is removed and added to either 6% aqueous trichloroacetic acid or ethanol. The cloudy reaction sample is cooled (4°C) for 15 minutes and then spun to pellet the precipitated serum proteins. The presence of the peptides is then determined by reversed-phase HPLC using stability-specific chromatography conditions.

The peptides of the present invention or analogs thereof which have CTL stimulating activity may be modified to provide desired attributes other than improved serum half life. For instance, the ability of the peptides to induce CTL activity can be enhanced by linkage to a sequence which contains at least one epitope that is capable of inducing a T helper cell response. Particularly preferred immunogenic peptides/T helper conjugates are linked by a spacer molecule. The spacer is typically comprised of relatively small, neutral molecules, such as amino acids or amino acid mimetics, which are substantially uncharged under physiological conditions and may have linear or

branched side chains. The spacers are typically selected from, e.g., Ala, Gly, or other neutral spacers of nonpolar amino acids or neutral polar amino acids. It will be understood that the optionally present spacer need not be comprised of the same residues and thus may be a hetero- or homo-oligomer. When present, the spacer will usually be at least one or two residues, more usually three to six residues. Alternatively, the CTL peptide may be linked to the T helper peptide without a spacer.

The immunogenic peptide may be linked to the T helper peptide either directly or via a spacer either at the amino or carboxy terminus of the CTL peptide. The amino terminus of either the immunogenic peptide or the T helper peptide may be acylated. Exemplary T helper peptides include tetanus toxoid 830-843, influenza 307-319, malaria circumsporozoite 382-398 and 378-389.

In some embodiments it may be desirable to include in the pharmaceutical compositions of the invention at least one component which primes CTL. Lipids have been identified as agents capable of priming CTL *in vivo* against viral antigens. For example, palmitic acid residues can be attached to the alpha and epsilon amino groups of a Lys residue and then linked, e.g., via one or more linking residues such as Gly, Gly-Gly-, Ser, Ser-Ser, or the like, to an immunogenic peptide. The lipidated peptide can then be injected directly in a micellar form, incorporated into a liposome or emulsified in an adjuvant, e.g., incomplete Freund's adjuvant. In a preferred embodiment a particularly effective immunogen comprises palmitic acid attached to alpha and epsilon amino groups of Lys, which is attached via linkage, e.g., Ser-Ser, to the amino terminus of the immunogenic peptide.

As another example of lipid priming of CTL responses, *E. coli* lipoproteins, such as tripalmitoyl-S-glycerylcysteinylserine (P₃CSS) I can be used to prime virus specific CTL when covalently attached to an appropriate peptide. See, Deres et al., *Nature* 342:561-564 (1989), incorporated herein by reference. Peptides of the invention can be coupled to P₃CSS, for example, and the lipopeptide administered to an individual to specifically prime a CTL response to the target antigen. Further, as the induction of neutralizing antibodies can also be primed with P₃CSS conjugated to a peptide which displays an appropriate epitope, the two compositions can be combined to more effectively elicit both humoral and cell-mediated responses to infection.

In addition, additional amino acids can be added to the termini of a peptide to provide for ease of linking peptides one to another, for coupling to a carrier support,

or larger peptide, for modifying the physical or chemical properties of the peptide or oligopeptide, or the like. Amino acids such as tyrosine, cysteine, lysine, glutamic or aspartic acid, or the like, can be introduced at the C- or N-terminus of the peptide or oligopeptide. Modification at the C terminus in some cases may alter binding characteristics of the peptide. In addition, the peptide or oligopeptide sequences can differ from the natural sequence by being modified by terminal-NH₂ acylation, e.g., by alkanoyl (C₁-C₂₀) or thioglycolyl acylation, terminal-carboxyl amidation, e.g., ammonia, methylamine, etc. In some instances these modifications may provide sites for linking to a support or other molecule.

The peptides of the invention can be prepared in a wide variety of ways. Because of their relatively short size, the peptides can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. See, for example, Stewart and Young, *Solid Phase Peptide Synthesis*, 2d. ed., Pierce Chemical Co. (1984), *supra*.

Alternatively, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes an immunogenic peptide of interest is inserted into an expression vector, transformed or transfected into an appropriate host cell and cultivated under conditions suitable for expression. These procedures are generally known in the art, as described generally in Sambrook et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, New York (1982), which is incorporated herein by reference. Thus, fusion proteins which comprise one or more peptide sequences of the invention can be used to present the appropriate T cell epitope.

As the coding sequence for peptides of the length contemplated herein can be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci et al., *J. Am. Chem. Soc.* 103:3185 (1981), modification can be made simply by substituting the appropriate base(s) for those encoding the native peptide sequence. The coding sequence can then be provided with appropriate linkers and ligated into expression vectors commonly available in the art, and the vectors used to transform suitable hosts to produce the desired fusion protein. A number of such vectors and suitable host systems are now available. For expression of the fusion proteins, the coding sequence will be provided with operably linked start and stop codons, promoter and terminator regions and usually a replication system to provide an expression vector for

expression in the desired cellular host. For example, promoter sequences compatible with bacterial hosts are provided in plasmids containing convenient restriction sites for insertion of the desired coding sequence. The resulting expression vectors are transformed into suitable bacterial hosts. Of course, yeast or mammalian cell hosts may also be used, employing suitable vectors and control sequences.

The peptides of the present invention and pharmaceutical and vaccine compositions thereof are useful for administration to mammals, particularly humans, to treat and/or prevent viral infection and cancer. Examples of diseases which can be treated using the immunogenic peptides of the invention include prostate cancer, hepatitis B, hepatitis C, AIDS, renal carcinoma, cervical carcinoma, lymphoma, CMV and condyloma acuminatum.

For pharmaceutical compositions, the immunogenic peptides of the invention are administered to an individual already suffering from cancer or infected with the virus of interest. Those in the incubation phase or the acute phase of infection can be treated with the immunogenic peptides separately or in conjunction with other treatments, as appropriate. In therapeutic applications, compositions are administered to a patient in an amount sufficient to elicit an effective CTL response to the virus or tumor antigen and to cure or at least partially arrest symptoms and/or complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on, e.g., the peptide composition, the manner of administration, the stage and severity of the disease being treated, the weight and general state of health of the patient, and the judgment of the prescribing physician, but generally range for the initial immunization (that is for therapeutic or prophylactic administration) from about 1.0 μ g to about 5000 μ g of peptide for a 70 kg patient, followed by boosting dosages of from about 1.0 μ g to about 1000 μ g of peptide pursuant to a boosting regimen over weeks to months depending upon the patient's response and condition by measuring specific CTL activity in the patient's blood. It must be kept in mind that the peptides and compositions of the present invention may generally be employed in serious disease states, that is, life-threatening or potentially life threatening situations. In such cases, in view of the minimization of extraneous substances and the relative nontoxic nature of the peptides, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these peptide compositions.

For therapeutic use, administration should begin at the first sign of viral

infection or the detection or surgical removal of tumors or shortly after diagnosis in the case of acute infection. This is followed by boosting doses until at least symptoms are substantially abated and for a period thereafter. In chronic infection, loading doses followed by boosting doses may be required.

5 Treatment of an infected individual with the compositions of the invention may hasten resolution of the infection in acutely infected individuals. For those individuals susceptible (or predisposed) to developing chronic infection the compositions are particularly useful in methods for preventing the evolution from acute to chronic infection. Where the susceptible individuals are identified prior to or during infection,
10 for instance, as described herein, the composition can be targeted to them, minimizing need for administration to a larger population.

The peptide compositions can also be used for the treatment of chronic infection and to stimulate the immune system to eliminate virus-infected cells in carriers. It is important to provide an amount of immuno-potentiating peptide in a formulation and mode of administration sufficient to effectively stimulate a cytotoxic T cell response.
15 Thus, for treatment of chronic infection, a representative dose is in the range of about 1.0 μg to about 5000 μg , preferably about 5 μg to 1000 μg for a 70 kg patient per dose. Immunizing doses followed by boosting doses at established intervals, e.g., from one to four weeks, may be required, possibly for a prolonged period of time to effectively
20 immunize an individual. In the case of chronic infection, administration should continue until at least clinical symptoms or laboratory tests indicate that the viral infection has been eliminated or substantially abated and for a period thereafter.

The pharmaceutical compositions for therapeutic treatment are intended for parenteral, topical, oral or local administration. Preferably, the pharmaceutical
25 compositions are administered parenterally, e.g., intravenously, subcutaneously, intradermally, or intramuscularly. Thus, the invention provides compositions for parenteral administration which comprise a solution of the immunogenic peptides dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine,
30 hyaluronic acid and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain

pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

5 In some embodiments it may be desirable to include in the pharmaceutical composition at least one component which enhances priming of CTL. Lipids have been identified as agents capable of enhancing priming of CTL *in vivo* against viral antigens. For example, palmitic acid residues can be attached to the alpha and epsilon amino groups of a Lys residue and then linked, e.g., typically via one or more linking residues
10 such as Gly, Gly-Gly-, Ser, Ser-Ser, or the like, to a synthetic peptide which comprises a class I-restricted CTL epitope. The lipidated peptide can be administered in saline or incorporated into a liposome emulsified in an adjuvant, e.g., incomplete Freund's adjuvant. In a preferred embodiment a particularly effective immunogen comprises palmitic acid attached to alpha and epsilon amino groups of Lys, which is attached via
15 linkage, e.g., Ser-Ser, to the amino terminus of a class I restricted peptide having T cell determinants, such as those peptides described herein as well as other peptides which have been identified as having such determinants.

As another example of lipid priming of CTL responses, *E. coli* lipoprotein such as tripalmitoyl-S-glycerylcysteinyl-serine (P₃CSS), can be used to prime virus
20 specific CTL when covalently attached to an appropriate peptide. See, Deres et al., *Nature* 342:561-564 (1989), incorporated herein by reference. Peptides of the invention can be coupled to P₃CSS, for example, and the lipopeptide administered to an individual to specifically prime a CTL. Further, as the induction of neutralizing antibodies can also be primed with P₃CSS conjugated to a peptide which displays an appropriate epitope, the
25 two compositions can be combined to more effectively elicit both humoral and cell-mediated responses to viral infection.

The concentration of CTL stimulatory peptides of the invention in the pharmaceutical formulations can vary widely, i.e., from less than about 0.1%, usually at or at least about 2% to as much as 20% to 50% or more by weight, and will be selected
30 primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

The peptides of the invention may also be administered via liposomes, which serve to target the peptides to a particular tissue, such as lymphoid tissue, or

targeted selectively to infected cells, as well as increase the half-life of the peptide composition. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In these preparations the peptide to be delivered is incorporated as part of a liposome, alone or in conjunction with a molecule which binds to, e.g., a receptor prevalent among lymphoid cells, such as monoclonal antibodies which bind to the CD45 antigen, or with other therapeutic or immunogenic compositions. Thus, liposomes filled with a desired peptide of the invention can be directed to the site of lymphoid cells, where the liposomes then deliver the selected therapeutic/immunogenic peptide compositions. Liposomes for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, e.g., liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, e.g., Szoka et al., *Ann. Rev. Biophys. Bioeng.* 9:467 (1980), U.S. Patent Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369, incorporated herein by reference.

For targeting to the immune cells, a ligand to be incorporated into the liposome can include, e.g., antibodies or fragments thereof specific for cell surface determinants of the desired immune system cells. A liposome suspension containing a peptide may be administered intravenously, locally, topically, etc. in a dose which varies according to, *inter alia*, the manner of administration, the peptide being delivered, and the stage of the disease being treated.

For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, one or more peptides of the invention, and more preferably at a concentration of 25%-75%.

For aerosol administration, the immunogenic peptides are preferably supplied in finely divided form along with a surfactant and propellant. Typical percentages of peptides are 0.01%-20% by weight, preferably 1%-10%. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of

such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute
5 0.1%-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. A carrier can also be included, as desired, as with, e.g., lecithin for intranasal delivery.

In another aspect the present invention is directed to vaccines which contain as an active ingredient an immunogenically effective amount of an immunogenic peptide as described herein. The peptide(s) may be introduced into a host, including
10 humans, linked to its own carrier or as a homopolymer or heteropolymer of active peptide units. Such a polymer has the advantage of increased immunological reaction and, where different peptides are used to make up the polymer, the additional ability to induce antibodies and/or CTLs that react with different antigenic determinants of the
15 virus or tumor cells. Useful carriers are well known in the art, and include, e.g., thyroglobulin, albumins such as human serum albumin, tetanus toxoid, polyamino acids such as poly(lysine:glutamic acid), influenza, hepatitis B virus core protein, hepatitis B virus recombinant vaccine and the like. The vaccines can also contain a physiologically tolerable (acceptable) diluent such as water, phosphate buffered saline, or saline, and
20 further typically include an adjuvant. Adjuvants such as incomplete Freund's adjuvant, aluminum phosphate, aluminum hydroxide, or alum are materials well known in the art. And, as mentioned above, CTL responses can be primed by conjugating peptides of the invention to lipids, such as P₃CSS. Upon immunization with a peptide composition as
25 described herein, via injection, aerosol, oral, transdermal or other route, the immune system of the host responds to the vaccine by producing large amounts of CTLs specific for the desired antigen, and the host becomes at least partially immune to later infection, or resistant to developing chronic infection.

Vaccine compositions containing the peptides of the invention are administered to a patient susceptible to or otherwise at risk of viral infection or cancer to
30 elicit an immune response against the antigen and thus enhance the patient's own immune response capabilities. Such an amount is defined to be an "immunogenically effective dose." In this use, the precise amounts again depend on the patient's state of health and weight, the mode of administration, the nature of the formulation, etc., but generally

range from about 1.0 μ g to about 5000 μ g per 70 kilogram patient, more commonly from about 10 μ g to about 500 μ g.mg per 70 kg of body weight.

In some instances it may be desirable to combine the peptide vaccines of the invention with vaccines which induce neutralizing antibody responses to the virus of interest, particularly to viral envelope antigens.

For therapeutic or immunization purposes, the peptides of the invention can also be expressed by attenuated viral hosts, such as vaccinia or fowlpox. This approach involves the use of vaccinia virus as a vector to express nucleotide sequences that encode the peptides of the invention. Upon introduction into an acutely or chronically infected host or into a non-infected host, the recombinant vaccinia virus expresses the immunogenic peptide, and thereby elicits a host CTL response. Vaccinia vectors and methods useful in immunization protocols are described in, e.g., U.S. Patent No. 4,722,848, incorporated herein by reference. Another vector is BCG (Bacille Calmette Guerin). BCG vectors are described in Stover et al. (*Nature* 351:456-460 (1991)) which is incorporated herein by reference. A wide variety of other vectors useful for therapeutic administration or immunization of the peptides of the invention, e.g., *Salmonella typhi* vectors and the like, will be apparent to those skilled in the art from the description herein.

Antigenic peptides may be used to elicit CTL *ex vivo*, as well. The resulting CTL, can be used to treat chronic infections (viral or bacterial) or tumors in patients that do not respond to other conventional forms of therapy, or will not respond to a peptide vaccine approach of therapy. *Ex vivo* CTL responses to a particular pathogen (infectious agent or tumor antigen) are induced by incubating in tissue culture the patient's CTL precursor cells (CTLp) together with a source of antigen-presenting cells (APC) and the appropriate immunogenic peptide. After an appropriate incubation time (typically 1-4 weeks), in which the CTLp are activated and mature and expand into effector CTL, the cells are infused back into the patient, where they will destroy their specific target cell (an infected cell or a tumor cell).

The peptides may also find use as diagnostic reagents. For example, a peptide of the invention may be used to determine the susceptibility of a particular individual to a treatment regimen which employs the peptide or related peptides, and thus may be helpful in modifying an existing treatment protocol or in determining a prognosis for an affected individual. In addition, the peptides may also be used to predict which

individuals will be at substantial risk for developing chronic infection.

The following example is offered by way of illustration, not by way of limitation.

Example 1

Identification of immunogenic peptides

Using the B7-like-supermotifs identified in the parent applications described above, sequences from potential antigenic sources including Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), Human Papilloma Virus (HPV), Human Immunodeficiency Virus (HIV), MAGE2/3, and Plasmodium were analyzed for the presence of these motifs.

Sequences for the target antigens were obtained from the current GenBank data base. The identification of motifs was done using the "FINDPATTERNS" program (Devereux et al., *Nucleic Acids Research* 12:387-395 (1984)). A computer search was carried out for antigen proteins comprising the B7-like-supermotif.

Table 5 lists peptides identified in this search. Accordingly, a preferred embodiment of the invention comprises a composition comprising a peptide of Table 5.

Other viral and tumor-related proteins can also be analyzed for the presence of these motifs. The amino acid sequence or the nucleotide sequence encoding products is obtained from the GenBank database in the cases of Prostate Specific antigen (PSA), p53 oncogene, Epstein Barr Nuclear Antigen-1 (EBNA-1), and c-erb2 oncogene (also called HER-2/neu).

In the cases of Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), and Human Immunodeficiency Virus (HIV) several strains/isolates exist and many sequences have been placed in GenBank.

For HBV, binding motifs are identified for the adr, adw and ayw types. In order to avoid replication of identical sequences, all of the adr motifs and only those motifs from adw and ayw that are not present in adr are added to the list of peptides.

In the case of HCV, a consensus sequence from residue 1 to residue 782 is derived from 9 viral isolates. Motifs are identified on those regions that have no or very little (one residue) variation between the 9 isolates. The sequences of residues 783 to 3010 from 5 viral isolates were also analyzed. Motifs common to all the isolates are identified and added to the peptide list.

Finally, a consensus sequence for HIV type 1 for North American viral

isolates (10-12 viruses) was obtained from the Los Alamos National Laboratory database (May 1991 release) and analyzed in order to identify motifs that are constant throughout most viral isolates. Motifs that bear a small degree of variation (one residue, in 2 forms) were also added to the peptide list.

5

The above examples are provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference.

Table 5

PEPTIDE AA	AA	SEQUENCE	SOURCE	B*0701
1021	9	FPFKYAAAF	B35 consensus peptid	0
1054	9	YPKVKQWPL	Y1 analog of 1054.05	0
1075	11	CILESCFRAVI	MAGE-1	0
1080	9	YPAEITLYW	B53 self peptide	0
1086	9	FAMPNFQTL	Cw3 consensus	0
1086	9	FAMPNFYTL	Cw3 consensus	0
1086	9	QPDDAVYKL	Cw4 consensus	0
1086	9	IPYPIVRKL	Cw6 consensus	1
1086	9	IPYPIVRSL	Cw6 consensus	1
1086	9	IPFPIVRYL	Cw6 consensus	0
1086	9	RYRPGTVAL	Histone H3.3	0
	9	MPRGVVVTL	B7 Nat. Processed	3
	10	LPENNVLSPL	p53. 26-35	0
	10	APAPAPSWPL	p53. 84-93	1
	11	SPALNKMFCQL	p53. 127-137	0
	9	GTRVRAMAI	p53. 154-162	0
	9	RPILTIITL	p53. 249-257	0
	10	LPPGSTKRAL	p53. 299-308	0
	9	SPQPKKKPL	p53. 315-323	0
	10	KPLDGEYFTL	p53. 321-330	0
	9	GSAHSSHL	p53. 361-369	0

WHAT IS CLAIMED IS:

1. A composition comprising an immunogenic peptide having a supermotif which allows the immunogenic peptide to bind more than one HLA molecule, the immunogenic peptide having between about 9 and about 10 residues;
5 a first conserved residue at the second position from the N-terminus being P; and
a second conserved residue at the C-terminal position being selected from the group consisting of M, I, and an aromatic residue.
- 10 2. The composition of claim 1, wherein the immunogenic peptide is selected from the group consisting of SEQ. ID. Nos. 1-21.
3. A method for inducing a CTL response in a patient, the method comprising administering to the patient a therapeutically effective dose of the
15 immunogenic peptide of claim 1.

1/2

B7-LIKE CROSS-REACTIVE MOTIF

YFW	STC					YFW			A
	1	2	3	4	5	6	7	8	
A				QN		DE	QN	DE	
DE						G	A		
G							G		
P									

+

-

* MOTIF IS COMPRISED OF ALL RESIDUES WHICH ARE "+" OR "-" FOR TWO OR MORE ALLELES.

FIG 1.

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2/2

B*070I										
++	RHK,YFW	RHK	G	RHK	QN	STC	P,A	L,M		
+	1	2	3	4	5	6	7	8	9	
-			P		A	P,DE				A
--	DE,P	DE	DE	DE,QN	G	QN	DE			Y,W
B*350I										
++	YFW				QN	YFW	A	Y,F		
+	1	2	3	4	5	6	7	8	9	
-										
--	A,G,P		QN		G	A,G,QN	DE			W
B*530I										
++	YFW		STC		G	YFW		F,W		
+	1	2	3	4	5	6	7	8	9	
-										
--	A,G,QN,P		QN		G	A,QN,P	DE			M
B*540I										
++	YFW	YFW,LIVM		LIVM	STC	LIVM	A,YFW	A		
+	1	2	3	4	5	6	7	8	9	
-			G							F,Y,M
--	G,DE,P		P	RHK	G,DE	QN	RHK	DE		W

FIG. 2.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/09234

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 38/03, 45/05
US CL : 424/184.1, 185.1, 277.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1, 185.1, 277.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Examiner's MHC filesElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Automated Patent Search (APS): key words: major histocompat7, binding motif, peptid7

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Gazz. Chim. Ital., Volume 97, No. 12, issued 1967, G. Bosisio et al., "Sintesis di analoghi della Fisalemina", pages 1848-1857, see peptide sequences on page 1848.	1
A	Current Opinion in Immunology, Volume 6, issued 1994, V. H. Engelhard, "Structure of peptides associated with MHC class I molecules", pages 13-23, see entire document.	1-3
A	Current Opinion in Immunology, Volume 2, issued 1989, J. B. Rothbard, "Major histocompatibility complex-peptide interactions", pages 99-105, see entire document.	1-3

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents as is indicated being obvious to a person skilled in the art

"A" document member of the same patent family

Date of the actual completion of the international search

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